JOURNAL OF BACTERIOLOGY, Mar. 1969, p. 1419-1425
Vol. 97, No. 3

Printed in U.S.A.

Characterization of a β-Galactosidase Formed Between a Complementary Protein and a Peptide Synthesized De Novo

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Received for publication 29 November 1968

In a cell-free system, φ80d*lac* can be transcribed, and the resulting ribonucleic acid can be translated to yield a product which interacts with an enzymatically inactive *z* protein to produce active enzyme. The inactive *z* protein is produced by *Escherichia coli* strain 21, which contains a deletion in the first part of the gene for β-galactosidase and appears to exist as a dimer. The enzyme formed in the cell-free system appears to be composed of one strain 21 *z* protein dimer and one newly synthesized polypeptide chain with a molecular weight of about 3×10^4. The estimated size of this complementing segment is in good agreement with Ullmann, Jacob, and Monod's estimate of the size of the α region of β-galactosidase. Using α fragments produced by autoclaving or guanidine treatments, we found that the active portion of α seems to be smaller than the full α region. We also found, using α produced by the autoclaving technique, that active dimer undergoes conversion to tetramer as the amount of α is increased. Evidently, the binding of α favors this conversion, but it is unlikely that the conversion of dimer to tetramer per se results in increased enzyme activity.

Previously, we described the cell-free synthesis of a segment of the polypeptide chain of the enzyme β-galactosidase of *Escherichia coli* (3, 14). The synthesis requires the presence of deoxyribonucleic acid (DNA) containing the gene for β-galactosidase and all of the components necessary for transcription of DNA and translation of the resulting messenger ribonucleic acid (mRNA). The α segment synthesized in this system is detected by complementation with a cell-free extract from *E. coli* strain 21, which carries an inactive β-galactosidase with a deletion in the α region. When the α fragment is synthesized by use of cell-free S-30 extracts from a *lac* deletion strain, complementation is carried out after the synthesis by mixing the incubation product with an extract of strain 21. Alternatively, an S-30 extract from strain 21 can be used in the cell-free system. In this case, the complementation occurs during the synthetic incubation period. The latter procedure results in considerably higher enzyme activity and is the method used in the experiments described here. The object of this study was to characterize the enzyme formed in the cell-free system. In the course of this work, we made some new observations about the enzymatic activity of subunits of β-galactosidase.

MATERIALS AND METHODS

Incubation conditions for synthesis. The incubation mixture contained, per ml: 40 μmoles of tris(hydroxymethyl)aminomethane (Tris)-acetate (*p*H 8.2), 1.25 μmoles of dithiothreitol, 50 μmoles of potassium acetate, 25 μmoles of ammonium acetate, 0.2 μmole of 19 ^{12}C-L-amino acids, 2 μmoles of adenosine-5'-triphosphate, 0.5 μmoles each of the 5'-triphosphates of cytidine, guanosine, and uridine, 18 μmoles of trisodium phosphoenolpyruvate, 100 μg of transfer RNA prepared according to the procedure of Zubay (13), 25 μg of pyridoxine monohydrochloride, 25 μg of nicotinamide adenine dinucleotide phosphate, 10 μg of *p*-aminobenzoic acid, 25 μg of flavine adenine dinucleotide; 13.4 μmoles of magnesium acetate; 6.7 μmoles of calcium chloride; 25 μg of folinic acid; 0.1 μmole of ^{14}C-leucine (specific activity, 10^6 counts per min per μmole; in some experiments, 0.08 mc of ^{3}H-leucine/ml of incubation mixture was used instead of ^{14}C-leucine), 6,500 μg of protein as S-30 extract prepared as described below, and 200 μg of DNA from the virus φ80d*lac*. All ingredients, except the S-30 extract and the folinic acid, were mixed together and preincubated for 3 min at 37 C with mild shaking. The folinic acid was added to the S-30 extract, and this mixture was allowed to stand, protected from light, in ice for 5 min. The S-30-folinic acid mixture was added to the preincubated ingredients, and then the reaction tubes were incubated for 60 min at 37 C with mild shaking.

In all experiments described here, the S-30 extract was prepared from *E. coli* strain 21 F'i^-, which carries the M-15 deletion (see reference 10 for a description of this deletion) in the α region of the *z* gene on both chromosome and episome. The *z* gene product from this strain has no enzyme activity and will be referred to as 21-protein. When an S-30 extract from strain 21 is used in the incubation mixture, the α segment synthesized complements with the 21-protein during the course of the incubation and can be detected by assaying for β-galactosidase activity.

In all experiments for cell-free synthesis, controls consisting of either complete system minus DNA or complete system plus deoxyribonuclease were run and analyzed for ^{14}C-leucine (or ^{3}H-leucine) incorporation and β-galactosidase activity.

Preparation of S-30 extracts. S-30 extracts were prepared according to the method of Nirenberg (8) with the following modifications. The cells were washed twice with buffer I (0.01 M Tris-acetate, *p*H 7.8, 0.014 M magnesium acetate, 0.06 M potassium chloride, and 0.006 M 2-mercaptoethanol) and then suspended in buffer II (buffer I with 0.001 M dithiothreitol in place of the 2-mercaptoethanol) in a ratio of 1.33 ml of buffer per g of cells. The cells were lysed in a French pressure cell, and 1 μmole of dithiothreitol per ml was added to the lysate. No deoxyribonuclease was added to the lysate. After two 30-min centrifugations at 30,000 $\times$ *g* and preincubation as described by Nirenberg, the extract was dialyzed against buffer III (buffer II with 0.06 M potassium acetate in place of the potassium chloride). The S-30 extract was rapidly frozen in 2- to 3-ml portions and stored at -85 C.

Preparation of ϕ80 dlac DNA. This procedure was carried out as described previously (5).

Sucrose gradients. In all cases except one, 5 to 20% linear sucrose gradients in 0.01 M sodium phosphate buffer (pH 7.3), 0.001 M magnesium acetate, and 0.14 M 2-mercaptoethanol were used. The exception was the experiment made to determine whether the enzyme was ribosome bound. In that case, the gradient buffer was 0.01 M Tris-acetate (*p*H 7.3), 0.01 M magnesium acetate, and 0.14 M 2-mercaptoethanol. Centrifugations were carried out at 24,000 rev/min in the SW 25.1 rotor of a Spinco model L-2 centrifuge at 5 C.

When recoveries of β-galactosidase activity from sucrose gradients were calculated, the apparent recoveries ranged between 50 and 75%. However, sucrose, at high concentrations, is a noncompetitive inhibitor of β-galactosidase (4). It was found that, when complemented enzyme was stored in 12.5% sucrose in phosphate buffer for 30 hr at 4 C, there was a loss of 30 to 60% of β-galactosidase activity (with a higher loss of activity when the initial enzyme activity was low). When this sucrose effect was taken into account, recovery of enzyme activity from the gradients was close to 100%.

β-Galactosidase assays. Assays on complete incubation mixtures and controls were carried out at the end of the synthetic period by mixing 0.2 ml of incubation mixture with 1.5 ml of *o*-nitrophenyl-β-D-galactoside (ONPG) solution (0.70 mg of ONPG per ml of buffer containing 0.1 M sodium phosphate, *p*H 7.3, and 0.14 M β-mercaptoethanol). The assay tubes were incubated for about 20 hr, and then 1 drop of acetic acid was added to each tube to precipitate the protein. This decreases the background absorption and prevents errors in optical density (OD) determinations due to turbidity. The tubes were quickly stirred, chilled in ice, and then centrifuged in the cold for 15 min at 2,000 $\times$ *g*. The supernatant liquid was transferred to a tube containing an equal volume of 1 M sodium carbonate. The optical density was determined at 420 nm by reading against a distilled water blank. The complete system has an OD_{420} at zero time of assay of about 0.04 read against distilled water. The zero time value has been subtracted from the values obtained after 20 hr of assay. Controls for spontaneous hydrolysis of ONPG were run by using 0.2 ml of water as the sample in the assay procedure. The Δ OD_{420}, even after 42 hr of incubation, was less than 0.01. All assays were done in duplicate.

Assays on sucrose gradients were carried out by mixing a sample from each gradient fraction with 1 ml of ONPG solution. These mixtures were incubated at 28 C until the desired color was obtained in the peak tubes (see figure legends for sample size and length of assay); then 1 ml of 1 M sodium carbonate was added and the optical density was determined at 420 nm. Controls for the spontaneous hydrolysis of ONPG were run, and the values were subtracted from the reported data. These controls consisted of samples of 5, 12.5, and 20% sucrose in gradient buffer plus 1 ml of ONPG. They were incubated for the same length of time as the gradient samples. These controls had an OD_{420} at zero time of assay of about 0.02 read against distilled water, and the increase in OD was always less than 0.01 OD unit after 42 hr of incubation. All assays were run until the OD in peak tubes was at least 10 times that in control tubes.

Preparation of α extracts by the autoclaving method (α_a). D. Zipser (*personal communication*) has found that by autoclaving suspensions of wild-type cells, one can obtain extracts with a very high α donor activity but no β-galactosidase activity. *E. coli* strain 49 (Hfr H, i^-, z^+, y^+, B_1^-) cells were suspended in extraction buffer (0.02 M Tris, 0.01 M ethylenediaminetetraacetate, 0.01 M sodium chloride, and 0.1 M 2-mercaptoethanol, *p*H 7.2) in a 1:1 (w/v) ratio. The mixture was heated to 120 C at a gauge pressure of 15 psi for 20 min, and then was cooled and centrifuged for 15 min at 2,000 $\times$ *g*. The supernatant liquid was retained and could be stored for weeks in a refrigerator with little loss of α activity.

Preparation of α extracts by the guanidine treatment (α_g). The α_g extracts were prepared according to the method of Ullmann, Jacob, and Monod (11), except for the preparation of the bacterial extract. We used an extract from *E. coli* strain 1 (z^-, i^-, B_1^-) which carries the same point mutation in the *z* gene as does the strain z^-1 used by Ullmann et al. (10, 11), prepared in the following manner. Frozen cells were thawed and washed in cold buffer I (described above). Cells were then suspended in a volume of

cold buffer I about equal to the weight of cells used and were lysed in a French pressure cell. The lysate was centrifuged for 30 min at 30,000 × *g* in the cold, and the supernatant liquid was used for guanidine treatment.

Assay of 21-protein. Fractions from sucrose gradients were assayed for 21-protein by mixing a 0.1-ml sample from each fraction with 0.04 ml of a preparation of α_a having a protein concentration of 12.5 mg of protein/ml. This was allowed to stand at room temperature for 45 min; then 1 ml of ONPG-containing solution was added, and β-galactosidase assays were carried out as described above. The 21-protein can be easily distinguished in a gradient which contains complemented enzyme. Since the 21-protein is present in great excess, addition of a high level of α_a results in a relatively high enzyme activity. The assay for 21-protein can therefore be stopped by addition of 1 M sodium carbonate before there is any significant breakdown of substrate by the complemented enzyme which is present in a very small quantity.

Protein assay. The modified Lowry method (9) was used to measure protein concentration with bovine serum albumin as a standard.

Assay of ^{14}C-leucine and ^{3}H-leucine incorporation. ^{14}C-leucine incorporation was measured as described by Lederman and Zubay (5). When ^{3}H-leucine was used, the samples were prepared in the same way as those containing ^{14}C-leucine, but counting was done on a Nuclear-Chicago windowless gas-flow counter.

RESULTS

Cell-free synthesis of a segment of β-galactosidase and detection by complementation with 21-protein. Previously (3), we described a cell-free system in which an operator-proximal segment of the enzyme β-galactosidase could be synthesized. Although the yields of enzyme activity from that early system were very low, there was abundant evidence (3, 14) that the appearance of the complementing segment was due to de novo synthesis. We were interested, therefore, in improving the synthetic capacity of the cell-free system so that we could more readily detect and study the products.

The system used in the experiments described here was developed by Lederman and Zubay (6). It gives up to 10-fold higher yields of enzyme activity than those reported previously. A unit of β-galactosidase is defined as the amount producing 1 μmole of *o*-nitrophenol per min at 28 C and *p*H 7.3. According to this definition, 1 ml of a typical incubation mixture used in these experiments contains about 0.22×10^{-3} units of enzyme. With the system described previously (3), the highest yield of enzyme in 1 ml of incubation mixture was about 0.05×10^{-3} units. This kind of quantitative estimate probably is not a good measure of the amount of β-galactosidase polypeptide the cell-free system is capable of producing. First of all, it assumes that the specific activity of the complemented enzyme is the same as that of wild-type enzyme—we do not know whether this is true. Second, in the experiments described here, we are only detecting those fragments which have complemented with 21-protein. Lederman and Zubay (6) found that, when synthesis is carried out with the use of an S-30 extract from a mutant which has a deletion of the entire *lac* operon, there is synthesis of a small amount of active polypeptide which approximates the β-galactosidase monomer in size. In the presence of 21-protein, this polypeptide is not detected, probably because the progression of synthesis is interrupted by complementation. From the sedimentation studies described here, it can be seen that the newly synthesized peptide which complements with 21-protein may correspond to about one-fifth of the β-galactosidase monomer.

Sedimentation studies on enzymes formed by complementation. Complementation studies were carried out with the use of extracts from *E. coli* strain 21 cells containing "21-protein" and α extracts prepared in three different ways, as described in Materials and Methods: (i) α_s refers to the segment synthesized in the cell-free system, (ii) α_a was prepared by the autoclaving technique of Zipser, and (iii) α_g was prepared by guanidine-HCl treatment, according to the method of Ullmann, Jacob, and Monod (11). The sedimentation behavior of the complemented enzymes was examined in the sucrose density gradients.

Enzyme formed by complementation between α_s and 21-protein. It was of interest to determine whether the enzyme produced in the synthetic system was ribosome-bound. This seemed possible in light of the report of Byrne and colleagues (1) of a complex containing DNA, ribosomes, RNA, and nascent protein which was formed during in vitro protein synthesis. Also, Zipser and Perrin (12) found that complementation between certain point mutants in the *z* gene occurred at a faster rate when one of the proteins was ribosome-bound than when both components were free.

During the incubation period for the synthesis of α in our system, a viscous precipitate developed which contained most of the DNA. When this was removed by centrifugation for 15 min at 2,000 × *g*, 98% of the β-galactosidase activity and 83% of the hot perchloric acid-insoluble radioactivity remained in the supernatant liquid (Table 1). A portion of this supernatant liquid was layered on a sucrose gradient and centrifuged for 188 min at 24,000 rev/min. The gradient was run through a flow cell in an LKB absorbance recorder, and the 1-ml fractions were collected and

TABLE 1. *β-Galactosidase activity and acid-insoluble ^3H-leucine produced in the synthetic system and derived fractions*

Fraction	^3H-leucine incorporated (counts per min per ml)	β-Galactosidase (units/ml)[a]
1. Incubation system product.....	4.78×10^5	0.201×10^{-3}
2. Supernatant liquid after 15 min, 2,000 × *g* centrifugation of 1....	3.98×10^5	0.197×10^{-3}
3. Supernatant liquid after 90 min, 158,000 × *g* centrifugation of 2....	2.55×10^5	0.143×10^{-3}
4. Same as 1 without DNA.....	0.53×10^5	0.003×10^{-3}
5. Same as 1 with 10 μg of deoxyribonuclease per ml.....	0.25×10^5	0.003×10^{-3}

[a] A unit of β-galactosidase is defined as the amount producing 1 μmole of *o*-nitrophenol per min at 28 C and pH 7.3.

assayed for β-galactosidase activity. It can be seen in Fig. 1 that most of the β-galactosidase activity remained near the meniscus of the gradient tube; very little enzyme activity was found in the ribosome or polysome region. The ribosome peak occurred in fraction 20, as indicated by the absorbance at 260 nm. This finding that the enzyme is not ribosome-bound was confirmed by the fact that, when the incubation mixture was centrifuged for 90 min at 40,000 rev/min to sediment the ribosomes, 71% of the enzyme activity (Table 1) remained in the supernatant fluid.

The sedimentation coefficient of the complemented enzyme from the cell-free system was estimated by the method of Martin and Ames (7) with the use of 21-protein as a marker (Fig. 2 and Table 2). A mean *S* value of 10.5 (with standard deviation of 0.4) was determined for the α_s-21 enzyme from nine gradients. An *S* value of 9.7 had been determined for 21-protein, by using alkaline phosphatase (6.3*S*) as an internal standard and by comparing it with wild-type β-galactosidase (16.6*S*) in a parallel gradient.

Enzyme formed by complementation between α_s and 21-protein. When the sedimentation behavior of α_s complemented with 21-protein was studied, an interesting phenomenon was observed. When the amount of α_s used in the complementation mixture was low, the enzyme had an *S* value of 9.7 (Fig. 3a). Within the limits of precision available to us, this is the same as the sedimenta

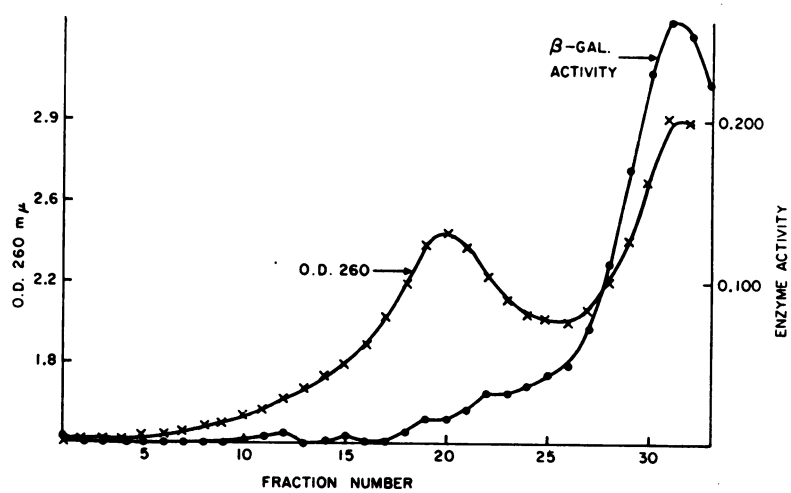

FIG. 1. *Sedimentation of the cell-free system product in a sucrose density gradient to determine whether the enzyme is ribosome-bound. A standard incubation mixture with a 21 S-30 was centrifuged for 15 min at 2,000 × g after the 60-min synthetic period. A 1-ml amount of supernatant liquid containing enzyme activity equivalent to about 0.20 × 10^{-3} units of β-galactosidase was layered on a 30-ml 5 to 20% linear sucrose gradient and centrifuged for 188 min. Enzyme activity was plotted in arbitrary units; one unit is equivalent to 10^{-4} units of β-galactosidase/ml.*

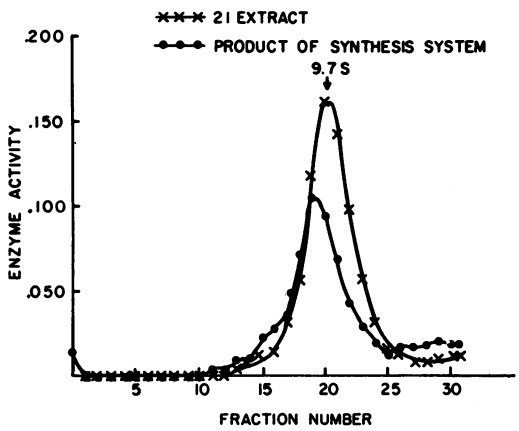

FIG. 2. *Sedimentation of the enzyme from the cell-free system compared to 21-protein. A standard incubation mixture with a 21 S-30 was prepared and incubated for 60 min; it was then centrifuged for 15 min at 2,000 × g. A 1-ml amount of supernatant liquid containing enzyme activity equivalent to about 0.22 × 10^{-3} units of β-galactosidase was layered on a 30-ml 5 to 20% sucrose gradient and centrifuged for 30 hr at 24,000 rev/min in the SW 25.1 rotor. Fractions (1.2 ml) were collected, and samples were taken from each and assayed for β-galactosidase activity and 21-protein. Enzyme activity was plotted in arbitrary units to allow comparison of the sedimentation profiles. An arbitrary unit of enzyme activity for the product of the cell-free system equals 5.6 × 10^{-5} units of β-galactosidase per 1.2-ml fraction. Enzyme activity for 21-protein is the β-galactosidase activity after complementation as described in Materials and Methods, and is equal to 0.114 units of β-galactosidase per 1.2-ml fraction.*

TABLE 2. *Sedimentation constants and estimated molecular weights for 21-proteins and enzymes formed by complementation*

Protein	Sedimentation constant	Molecular wt (daltons)
β-Galactosidase.....	16.6[a]	5.38 × 10^{5}[a]
21-Protein (M-15 protein).....	9.7	2.4 × 10^{5}
Enzyme from cell-free system.....	10.5	2.7 × 10^{5}
21-Protein complemented with α_s.....	9.7	2.4 × 10^{5}
21-Protein complemented with α_g.....	9.9	2.4 × 10^{5}

[a] Values taken from Craven, Steers, and Anfinsen (2).

tion coefficient of 21-protein. With a higher level of α_s, two enzyme peaks were seen in the sucrose density gradient, one at 9.7S and another at 16S (Fig. 3b). When the level of α_s was further raised, the 16S peak predominated (Fig. 3c).

To find whether there was any difference in enzyme activity between the two active species found, various amounts of α_s were complemented with excess 21 extract and the β-galactosidase activity was assayed (Fig. 4). The range of the ratio of α_s to 21 extract extends from below that where a single 9.7S species is found to the point where the 16S species predominates. The amount of enzyme formed in the presence of excess 21-protein was directly proportional to the amount of α_s added over the entire range studied.

Enzyme formed by complementation between α_g and 21-protein. Complementation was studied only at low levels of α_g. The resulting enzyme was found to have a sedimentation constant very close to that of 21-protein (Table 2).

DISCUSSION

In this paper, we have been concerned with the nature of the complemented enzyme formed between the synthesized de novo polypeptide fragment called α_s and 21-protein with which it combines to give β-galactosidase activity. We have also looked at the sedimentation behavior of complemented enzymes formed by the interaction of 21-protein with α extracts prepared by autoclaving or guanidine treatment. The molecular weights of various molecular species have been estimated by the equation used by Martin and Ames (*see* Table 2), which related sedimentation constants and molecular weights for proteins with an approximately spherical shape. We recognize the approximate nature of this calculation for the β-galactosidase derivatives in question. However, the sedimentation data were consistent and seem to be sufficiently clear to warrant a straightforward interpretation. The 21-protein has an estimated molecular weight about 10% less than that calculated for the β-galactosidase dimer. Since the deletion in strain 21 is about 10% of the total polypeptide chain of normal β-galactosidase (10, 11), it seems likely that 21-protein exists as a dimer. The enzymes formed between low levels of α and 21-protein have molecular weights of from 0 to 12% higher than the 21-protein, depending upon the way in which the α is prepared, suggesting that these enzymatically active molecules exist as complexes between the 21-dimer and the much smaller α peptide.

The amount of active enzyme formed in the presence of excess 21-protein is directly proportional to the amount of α over the entire range studied. This linear dependence on concentration suggests that the active enzyme requires one α per 21 dimer. Apparently, the α_s-containing 21 dimer can interact to form tetramer (16S) if present at sufficiently high concentration, but there is no indication that tetramer formation

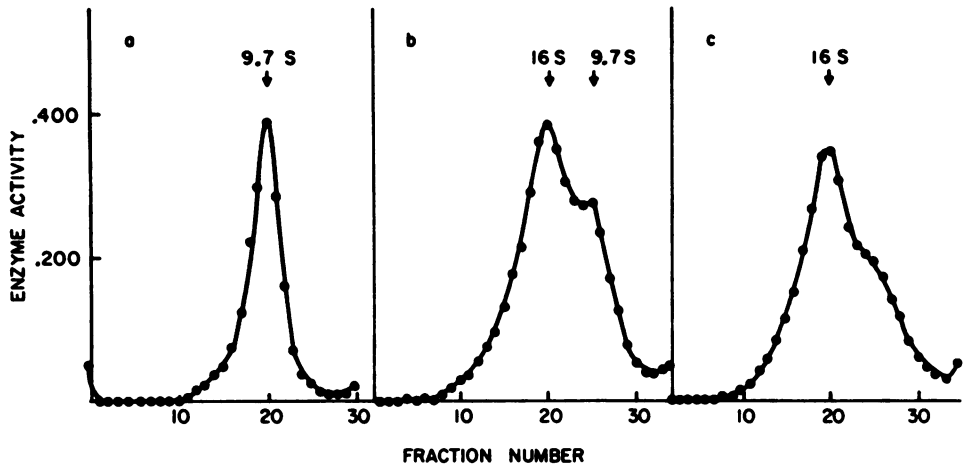

FIG. 3. *Effect of different levels of α_s in the complementation mixture on the sedimentation behavior of the complemented enzyme. The α_s extract (12.5 mg of protein/ml) and 21 extract (28 mg of protein/ml) were mixed in the proportions indicated below and were allowed to stand for 45 min at room temperature. Samples (0.5 or 1.0 ml) were layered on 30-ml 5 to 20% sucrose gradients and centrifuged at 24,000 rev/min in the SW 25.1 rotor. Fractions (1 ml) were collected, and β-galactosidase assays were carried out. Mixtures were as follows: (a) 1.0 part α_s, 9 parts 21 extract; centrifuged for 28 hr; (b) 2.2 parts α_s, 9 parts 21 extract; centrifuged for 22 hr; (c) 3.0 parts α_s, 9 parts 21 extract; centrifuged for 22 hr.*

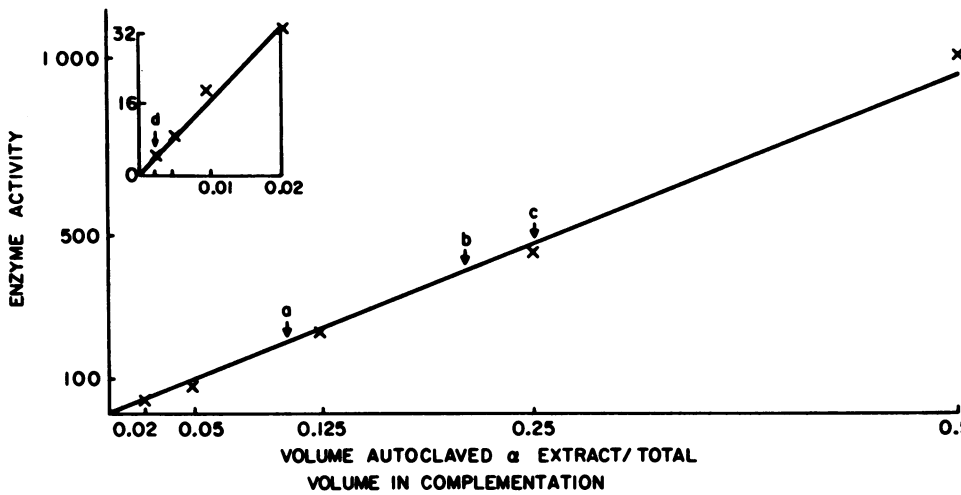

FIG. 4. *Relationship between β-galactosidase activity and concentration of α_s in the complementation mixture. Dilutions of α extract (12.5 mg of protein/ml) prepared by the autoclaving method were made in an autoclaved cell extract of approximately equal total protein concentration from a lac deletion strain of E. coli. A 0.1-ml amount of each α_s dilution was mixed with 0.1 ml of 21 extract (28 mg of protein/ml), allowed to stand for 1 hr at room temperature, and then assayed for β-galactosidase activity. Enzyme activity equals OD_{420} (per minute per milliliter × 100). Points a, b, and c, can be roughly correlated with the enzyme species a, b, and c in Fig. 3. Point d indicates the level of enzyme activity found in the incubation system.*

results in greater enzyme activity. Otherwise, we would see a break in the curve of Fig. 4 in the region where transition between dimer (9.7*S*) and tetramer (16*S*) takes place. It also seems very likely that the enzyme formed in the cell-free system contains one α_s per 21 dimer, since a very small amount of enzyme activity (*see* point d in Fig. 4) is formed in the presence of a vast excess of 21 dimer. This idea is also supported by the fact that the amount of enzyme activity is propor-

tional to the amount of φ80d*lac* DNA used in the cell-free system (6; *unpublished observation*). It is of great interest that the enzyme formed in the cell-free system and the enzyme formed between low levels of α_a or α_g have *S* values which suggest that they exist as α-containing 21 dimers. Previously, only the tetramer of β-galactosidase has been found to have enzyme activity (11).

When the molecular weights of the complemented enzymes were estimated by the formula of Martin and Ames (7), we found that the enzyme formed by complementation between the α segment synthesized in our system and 21-protein has a molecular weight of about 2.7×10^5. When this is compared to the molecular weight of 2.4×10^5 for uncomplemented 21-protein, it suggests that the α segment produced in the cell-free system has a molecular weight of about 3×10^4. Ullmann, Jacob, and Monod (10) defined the α region of β-galactosidase as "an operator-proximal segment of the gene, extending from the operator to about one-fifth or one-quarter of the genetic length." Assuming that the monomer (molecular weight, 135,000) length is proportional to the genetic length, the α region would correspond to a molecular weight of 27,000 to 33,750. Our estimate is in reasonable agreement with this.

Since the enzyme formed by complementation between 21-protein and the α fragments produced by autoclaving or guanidine treatment have *S* values which are the same as or close to that of uncomplemented 21-protein, these fragments may be considerably smaller than the full α region as defined by Ullmann et al. (10). This suggests that the entire α region is not needed to produce enzyme activity when complexed with 21-protein. Work is in progress to isolate the de novo synthesized α_s fragment, so that its size and other properties can be studied in greater detail.

ACKNOWLEDGMENTS

We thank David Zipser for providing the details of his autoclaving technique for preparing α extract and also for giving us many of the bacterial strains used.

This investigation was supported by Public Health Service grant GM 12768-3 from the National Institute of General Medical Sciences and by grant GB 7597 from the National Science Foundation. J. K. D. was a predoctoral fellow of the National Science Foundation during the course of this work.

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